Doping reversed-phase media for improved peptide purification

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ABSTRACT

The purification of therapeutic peptides is most often performed using one or more reversed phase chromatography steps. This ensures high purities while keeping the costs of purification under control. In this paper, a doped reversed phase chromatographic material is tested and compared to traditional reversed phase materials. The doping consists of adding limited amounts of ion exchange ligands to the surface of the material to achieve orthogonal separation and increase the non-hydrophobic interactions with the surface. These ionic groups can either be attractive (opposite charge), or repulsive (same charge) to the peptide. The benefit of this new doped reversed phase material is shown through increases in selectivity in diluted conditions and yield and productivity in overloaded (i.e. industrial) conditions. It is the conjectured that all performance characteristics should increase using repulsive doping groups, whereas these characteristics should decrease when using attractive doping groups. This conjecture is shown to be true through several examples, including purifications of industrially relevant peptide crudes, in industrially relevant conditions. Moreover, the effect of ionic strength and organic modifier concentration was explored and shown to be in line with the expected behavior.

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1. Introduction

In the last few years, an increased interest in peptides, for example as therapeutic agents, has led to an intensified demand in purification methods of these products. An important technique used in these purifications is chromatography, in particular, reversed phase chromatography (RPC). While RPC has proven to be a reliable, efficient and safe technique, its high cost has driven the development of ever more efficient purification techniques. Moreover, in order to increase the yield and productivity of a given separation, the concept of orthogonality, i.e. separation according to two or more types of interactions (for example hydrophobic and electrostatic), is usually introduced in different chromatographic steps. The efficacy of orthogonal chromatographic separations is well known, and is almost always applied in industrial scale chromatography [1,2].

Combining the need for new and improved types of RPC stationary phases and the concept of orthogonality, an innovative, reversed phase based, ion-exchange doped mixed mode material (doped reversed phase, DRP) has been developed. This different stationary phase exhibits orthogonality in a single material (i.e. in

a single chromatographic step) by the use of two types of ligands: first, the reversed phase ligands, which display the hydrophobic interactions; and second the ion exchange ligands, in doping quantities, which display the electrostatic interactions [3].

While most mixed mode materials carry both types of interactions on a single ligand, this material uses two separate ligands, each exhibiting its own type of interaction. This type of mixed mode material is sometimes called stochastic mixed mode (see Fig. 1) [4,5].

By having two distinct ligands, the surface concentration of each can be very precisely chosen. In this case, the ion exchange ligand density is much lower than that of the reversed phase ligands. The ion exchange (IEX) ligands are therefore said to be doping, whereas the material is defined as a doped reverse phase material. This is in contrast with most mixed mode materials where the interaction types are equally distributed along the surface. Additionally, most, if not all, mixed mode materials show only attractive interactions, whereas the doping IEX groups in DRP can be both used as attractive ligands (ligand and analyte have opposite charge) or as repulsive ligands (ligand and analyte have same charge). These modes are called attractive–attractive, or attractive (RP ligands) – repulsive (IEX ligands) respectively.

A somewhat similar RPC material is the CSH phase by Waters [6]. While this material also contains positively charged groups, along with the typical RP ligands, several differences with the DRP
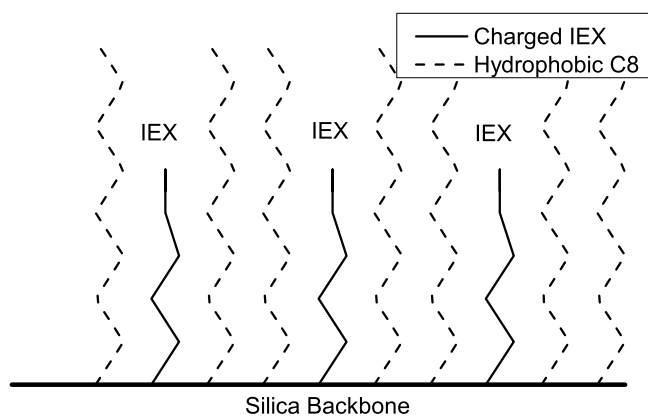
Fig. 1. Schematic of RPC surface doped with IEX groups.

exist. First, these charged groups appear to be directly part of the backbone, and are not added as a second ligand [7–9]. Second, the charge density of the positively charged groups on the CSH phase is 0.003 C/m^2 [10], whereas the minimal charge density on the DRP material is about 0.015 C/m^2 (five times as much). Other than the CSH, nothing comparable to the DRP was found in literature.

In this paper, the theory behind the design of the DRP material is discussed. In addition, the steps taken to design both the material and the purification step are discussed in detail. Several examples of improved performance, using the newly developed material, both in diluted and overloaded conditions, are then discussed.

2. Stationary phase design and functionality

2.1. Hydrophobic subtraction model

The hydrophobic subtraction model developed by Snyder et al. [11] accounts for the most commonly observed interactions between a solute and a RP material. These include hydrophobic interactions, steric resistance, hydrogen bonding and ion-exchange activity (pH dependent). An important result discussed in [11] is that a near linear correlation exists among the retention factors of many solutes (mainly exhibiting hydrophobic interactions) on different RP materials. This indicates that the hydrophobic interactions correlate between RP materials, with the other effects accounting for the "largely non-experimental error" scatter. As such, the difference in selectivity between RP materials is due largely to the non-hydrophobic interactions.

2.2. Doped reversed phase design concepts

In order to exhibit alternative selectivity, the DRP material was doped with ionic groups. As described above, if these dopants are of the same charge sign as the analytes, the material is used in attractive–repulsive mode. On the other hand, if the dopants and the analytes have opposite charges, the material is used in attractive–attractive mode.

RPC separations work on the basis of hydrophobicity: more hydrophobic components are more retained on a column than less hydrophobic components. Doping an RPC phase with ionic ligands adds an orthogonal separation capacity. It is important to point out that highly charged peptides are less hydrophobic (simply by containing more charged and therefore less hydrophobic amino acids). Inversely, more hydrophobic peptides are less charged. This allows both the attractive–attractive and attractive–repulsive modes of the DRP material to be described theoretically.

In the attractive–attractive mode, an additional interaction is added, which increases the retention of peptides on a column.

However, the most charged (least hydrophobic) peptides receive the biggest boost in retention. On the other hand, the most hydrophobic peptides receive a much smaller boost in retention. Considering a three way separation, with a product peptide pool P, a weakly retained (on RPC) impurity W and a strongly retained (on RPC) impurity pool S, an imaginary separation can be made. Pool W would see its retention increased the most, whereas pools P and S would only have small to inexistent increases in retention. This leads to an undesirable condition as the pools would converge to the same point. Moreover, the increased retention time would lead to broader peaks, effectively reducing the resolution. This mode is schematically shown in Fig. 2. A more rigorous description of the behavior of peptides in attractive–attractive mode is presented in [12].

On the other hand, in the attractive–repulsive mode, the retention is decreased due to the repulsive groups. Considering the same separation as above, the pool W would have its retention decreased the most, whereas pool P would only see a small decrease in retention. Pool S would not experience a decrease in retention. This leads to peak positions being further apart, which means better separation. Furthermore, since the peaks are less retained, they will be less broad thereby increasing the resolution. Finally, the separation will happen faster than in the RPC material. Again, the effect of using DRP in attractive–repulsive mode is shown in Fig. 2.

Another effect of the doping groups is due to modifier gradients. In gradient conditions, organic modifier concentrations go from low to high. This leads the dielectric constant of the mobile phase to go from high to low (see [13] for the dielectric constant of water–acetonitrile mixtures). This means the Debye Hückel length (length at which an electrostatic effect is meaningful) decreases along the gradient. The larger this length the larger the electrostatic effect. This has particular advantages for the attractive–repulsive mode, as the longer a component is retained, the less repulsion it experiences. The pool W would therefore be subject to much more repulsion than the pool S, thereby increasing the separation performance.

As the DRP materials are expected to perform best in attractive–repulsive mode, it will be discussed in most detail. In this case, since the doping groups are solely repulsive, they would only decrease the interaction strength between the analyte and the reversed phase backbone of the DRP material. As such, in constant ionic strength and pH, the material is expected to behave like a RPC material. This means that there are no new or complicated effects to take into consideration. In essence, the RPC hydrophobic interactions would be modulated by modifier type and concentration [14–17], ionic strength [18] and RPC ligand type [14]. The strength of the repulsive ionic interactions can in turn be modulated by ionic strength, pH, IEX ligand type and density [19], and modifier type and concentration [20]. It is of course evident that the nature of the analyte plays an elemental role in its retention behavior [14,19].

In the following sections, operating the DRP material in attractive–repulsive mode will be shown to give the best results.

3. Doped reversed phase materials

Several DRP materials were produced by Zeochem AG (Uetikon am See, Switzerland). All the materials used a commercially available silica gel as the base support (namely ZEOsphere 100 10 μm from Zeochem AG). Two ligands were used to functionalize the surface: C8 ligands were used as the base hydrophobic contributor; and either quaternary amines or sulfonate groups as the doping IEX groups [3,12]. The choice between the quaternary amine and sulfonate modified DPR material was done based on the analyte pI and the buffer pH. At buffer pH values below analyte pI (analyte positively charged), the quaternary amine acts as a repulsive group (attractive–repulsive mode), whereas the sulfonate behaves as an